Available online at http://ajol.info/index.php/ijbcs

Int. J. Biol. Chem. Sci. 7(4): 1441-1451, August 2013

International Journal of Biological and Chemical Sciences

ISSN 1991-8631

Original paper

http://indexmedicus.afro.who.int

Chemical composition and antifungal activity of *Piper capense* oil against mycotoxigenic *Aspergillus, Fusarium* and *Penicillium* species

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ABSTRACT

Hydro-distilled essential oil from *Piper capense* (L. f.) growing in Kenya was analyzed by gas chromatography mass spectrometry (GC-MS) and evaluated for antifungal activity. The oil was dominated by sesquiterpene hydrocarbons (43.9%) with δ-cadinene (16.82%), β-bisabolene (5.65%) and bicyclogermacrene (3.30%). There was appreciable quantity of monoterpene hydrocarbons (30.64%) including β-pinene (7.24%) and α-phellandrene (4.76%). Arylpropanoids (8.64%) was found in reasonable quantity having myristicin (4.26%) as its major constituent. A total of ten, fourteen and sixteen mycotoxigenic species of *Aspergillus*, *Fusarium* and *Penicillium* respectively were assayed. The oil showed strong antifungal activity against these fungi with Minimum Inhibition Concentration (MIC) ranging from 33.1 to 265 mg/ml. These results show that the oil has antifungal activities against fungi that are producers of poisonous mycotoxins found in foods and therefore can be used in food preservation systems to inhibit the growth of moulds and retard subsequent mycotoxin production.

Keywords: Essential oil, inhibition zone, moulds, mycotoxigenic fungi.

INTRODUCTION

Moulds are known to destroy 10 to 30% of the total yield of crops thus lower their nutritional and resale value (Agrios, 1997; Fandohan, 2003). They produce mycotoxins that contaminate about 25% of the agricultural crops worldwide and are a source of morbidity and mortality throughout Africa, Asia and Latin America (Smith et al., 1994). Consumption of mycotoxin contaminated commodities is related to several acute and chronic diseases in human and animals (Bhat and Miller, 2010) and the diseases or physiological abnormalities resulting from exposure to mycotoxins are known as "Mycotoxicosis". Mycotoxins are - known to cause acute liver damage, liver cirrhosis, induction of tumours and attack on the central nervous system, skin disorders and hormonal effects (Oguz et al., 2003; Makun et al., 2009;

DOI : http://dx.doi.org/10.4314/ijbcs.v7i4.2

Negedu et al., 2011). The five agriculturally important toxins from moulds are aflatoxins, fumonisins, ochratoxins, zearalenone and deoxynivalenol. These mycotoxins are known to be either carcinogenic (aflatoxin B1, ochratoxin and fumonisin B1); oestrogenic (zearalenone), neurotoxic (fumonisin B1) nephrotoxic (ochratoxin), dematotoxic (trichothecenes); immunosuppressive (aflatoxin B1, ochratoxin A, T-2 toxin) (Bankole and Adebanjo, 2003; Jestoi et al., 2004; Coronel et al., 2010).

Most mycotoxicoses are caused by the common and widespread moulds, namely *Aspergillus*, *Penicillium* and *Fusarium.* The *Aspergillus* species produce aflatoxins, ochratoxins, fumitoxins and gliotoxins, with aflatoxins being the most economically important mycotoxins. The *Fusarium* species are frequently associated with preharvest contaminated cereals and produce a class of mycotoxins known as fumonisins. *Penicillium* species produce a wide variety of mycotoxins that include ochratoxins (a property it shares with the *Aspergillus* species), citrinin (CIT), cyclopiazonic acid (CPA), patulin (PAT), penicillic acid (PIA) and roquefortine C (RQC) among others (Bernhoft et al., 2004).

There is an increasing interest to obtain alternative antimicrobial agents from natural sources for use in food preservation systems because the widespread use of synthetic preservatives has led to the appearance of resistant microorganisms (Gibbons, 1992; Akinpelu, 2001). It has been established that essential oils inhibit microbial growth (Matasyoh et al., 2007, 2009) and there is considerable interest in using them to preserve grains by retarding fungal growth and associated mycotoxin production (Bullerman et al., 1977; Chatterjee, 1989). The genus Piper (Piperaceae) is distributed largely in tropical and subtropical regions of the world and comprises of an estimated 2000 species (Gurb-Fakim, 2006). They are often shrubs, herbs or lianas commonly found in forest undergrowth (Jaramillo and Manos, 2001). The widespread ethnomedical use of piper species has led to an increased interest in the search for active compounds from these species, and it has been found that many of these plants contain a number of biological activities (Koroishi et al., 2008). *P. capense* has been reported to show antifungal (Samie et al., 2010) and antibacterial (Steenkamp et al., 2007) activities.

In the search for biodiversity resources with utilizable activity, we evaluated the antifungal activity of the essential oil of *Piper capense* against the mycotoxigenic species of *Aspergillus*, *Fusarium* and *Penicillium* genera isolated from maize.

MATERIALS AND METHODS

Plant material

Fresh plant materials of *P. capense* were collected from the equatorial rainforest in Kakamega, Kenya. A voucher specimen was deposited at the Department of Biological Sciences, Egerton University, Kenya.

Isolation of essential oils

Fresh whole plant materials of *P. capense* were subjected to hydro-distillation in a modified Clevenger-type apparatus for a minimum of 4 h. The essential oil was obtained in a yield of 0.2% w/w after drying over anhydrous sodium sulphate (Na_2SO_4).

GC, GC-MS analysis

Samples of essential oils were diluted in methyltert-butylether (MTBE) (1:100) and analysed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS (‘Restek’) (30m × 0.25 mm i.d., 0.25 μm film thickness) fused-silica capillary column. Helium (at 0.8 ml/min) was used as a carrier gas. Samples were injected in the split mode at a ratio of 1:10 – 1: 100. The injector was kept at 250 ^{o}C

and the transfer line at 280 ^{o}C. The column was maintained at 50 ^{o}C for 2 min and then programmed to 260 ^{o}C at 5 ^{o}C/min and held for 10 min at 260 ^{o}C. The MS was operated in the EI mode at 70 eV, in m/z range 42-350. Identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) and supplemented by Wiley and QuadLib 1607 GC – MS libraries. The relative proportions of the essential oil constituents are expressed as percentages obtained by peak area normalization, all relative response factors being taken as one.

Test fungi

Maize samples were collected from various households and market centers in Western Kenya. The surface-sterilized - grains were plated on Czapek Dox and potato dextrose agar containing 7.5% sodium chloride and 1gm streptomycin sulphate (for 1 litre of media). The plates were incubated at 25 ^{o}C and monitored daily for fungal growth for seven days. Identification of the moulds was done based on morphological and cultural characteristics using taxonomic keys (Pitt, 1979; Nelson et al., 1983; Kozakiewicz, 1989; Klich, 2002). Target moulds were sub-cultured to obtain pure single-spore cultures.

Antifungal assays

Paper disc diffusion inhibition test was used to screen for antimicrobial activity of the essential oil as described by Souza et al. (2005). One hundred microliters of mould suspension (approximately 10^{6} spores/ml) was uniformly spread on sterile potato dextrose agar media in petri dishes. Sterile sensitivity discs were soaked with 10 µl of the essential oil and placed at the center of the inoculated culture plates. The plates were incubated at 25 ^{o}C for 7–10 days. At the end of the incubation period, diameters of the inhibition zones were measured to the nearest millimeter (mm). Nystatin discs (100 µg) were used as the reference standard. The minimum inhibitory concentration (MIC) was determined using the paper disc diffusion method as described above. Serial dilutions of the essential oil were done using dimethyl sulfoxide (DMSO) which was also used as the negative control.

Data presentation

The data on inhibition zones were computed using Microsoft Office Excel 2003 to derive means and standard deviations.

RESULTS

Fifty two compounds constituting 93.6% of the total oil were identified. The constituents identified by GC-MS analysis, their retention times and area percentages are summarized in Table 1. The oil was dominated by 43.9% sesquiterpene hydrocarbons, and sesquiterpene fraction had a high percentage of δ-cadinene (16.82), β-bisabolene (5.65%), bicyclogermacrene (3.30%), β-cubebene, (E)-caryophyllene (2.85%), and germacrene B, (2.21%). The oil was also characterized by an appreciable amount of monoterpene hydrocarbons (30.64%) of which the main constituents were β-pinene (7.24%), α-phellandrene (4.76%), α-pinene (3.94%), sabinene (3.84%), limonene (3.06%), β-phellandrene (2.50%) and *p*-cymene (2.00%). Oxygenated monoterpenes and sesquiterpenes accounted for 4.30% and 6.12% of the oil, respectively. It also had a significant amount of arylpropanoids (8.64%), whose major constituent is myristicin (4.26%).

The oil showed antifungal activity against mycotoxigenic species from the three genera, namely; *Aspergillus*, *Fusarium* and *Penicillium*. These activities are summarized in Tables 2–4. The highest antifungal activities of the oil were observed against

Aspergillus species with the highest inhibition zone of 28.3 mm for *A. niger* and a corresponding MIC of 33.1 mg/ml. This was followed by *A. wentii* and *A. ochraceous* with inhibition zones of 20.3 and 18.7 mm respectively and an MIC value of 33.1 mg/ml for both of them. These inhibition zones were comparable to those of the reference standard nystatin. The most resistant *Aspergillus* species were *A. flavus* and *A. parasiticus* both with an MIC value of 132.5 mg/ml. Moderate antifungal activities were observed against *A. versicolor*, *A. ustus*, *A. terreus* and *A. tamari* which had a similar MIC value of 66.3 mg/ml (Table 2).

Table 1: Chemical composition of *Piper capense* oil.

Monoterpenes	**KI**	**%**		**KI**	**%**		**KI**	**%**
			***trans*-p-Menth-2-en-1-ol**	**1147**	**0.10**	**Germacrene D**	**1491**	**1.52**
α-Thujene	918	0.31	Camphor	1151	0.67	β-Selinene	1498	1.30
α-Pinene	926	3.94	Terpinen-4-ol	1187	0.07	Bicyclogermacrene	1505	3.30
Camphene	943	0.10	Cryptone	1192	0.04	α-Muurolene	1508	0.49
Sabinene	969	3.84	*trans*-Piperitol	1216	0.19	β-Bisabolene	1518	5.65
β-Pinene	975	7.24	Piperitone	1260	0.30	δ-Cadinene	1528	16.82
Myrcene	987	0.52	Bornyl acetate	1290	0.10	Germacrene B	1569	2.21
α-Phellandrene	1006	4.76	Anethole	1293	0.05	**Total**		**43.9**
para-Cymene	1025	2.00	Safrole	1296	0.05	**Oxygenated sesquiterpenes**		
Limonene	1030	3.06	**Total**		**4.30**	Spathulenol	1587	2.36
β-Phellandrene	1032	2.50	**Sesquiterpene hyrocarbons**			Caryophyllene oxide	1592	0.48
(Z)-β-Ocimene	1037	0.38	α-Cubebene	1355	1.01	5-epi-7-epi-α-Eudesmol	1617	1.78
(E)-β-Ocimene	1048	1.80	α-Copaene	1385	1.54	Shyobunol	1706	1.50
γ-Terpinene	1060	0.04	β-Bourbonene	1392	0.42	**Total**		**6.12**
trans-Sabinene hydrate	1072	0.10	β-Cubebene	1397	3.27	**Arylpropanoids**		
Terpinolene	1089	0.05	(E)-Caryophyllene	1429	2.85	Asaricin	1503	1.61
Total		**30.64**	γ-Elemene	1438	0.15	Myristicin	1530	4.26
Oxygenated monoterpenes			(Z)-β-Farnesne	1461	0.32	Elemicin	1554	1.08
2-Nonanone	1093	0.08	α-Humulene	1465	0.85	Apiole	1683	1.69
Linalool	1104	2.40	allo-Aromadendrene	1469	0.73	**Total**		**8.64**
cis-p-Menth-2-en-1-ol	1129	0.25	γ-muurolene	1484	1.47	Total percentages		**93.60**

Table 2: Antifungal activity of *P. capense* oil on ten *Aspergillus* species after seven days of inoculation.

Fungus	Inhibition zones (mm)						MIC mg/ml
	Essential oil, µg x 10^2					Nystatin	
	106.00	53.00	26.50	13.25	6.63	100 µg	
A. flavus	10.3 ± 0.58	7.3 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	24 ± 0.00	132.5
A. parasiticus	7.3 ± 0.58	6.3 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	20 ± 1.41	132.5
A. niger	28.3 ± 1.53	16.3 ± 1.15	9.7 ± 0.58	7.7 ± 0.60	6 ± 0.00	22 ± 1.41	33.1
A. fumigatus	11 ± 1.00	10 ± 0.00	8 ± 0.00	7 ± 0.00	6 ± 0.00	24 ± 2.82	33.1
A. ochraceus	18.7 ± 2.08	16.7 ± 0.58	11 ± 1.20	8.3 ± 0.60	6 ± 0.00	16 ± 1.41	33.1
A. versicolor	14.7 ± 0.58	9.3 ± 0.58	8 ± 0.00	6 ± 0.00	0 ±0.00	24 ± 1.41	66.3
A. wentii	20.3 ± 0.58	17 ± 1.00	11 ± 0.58	7.3 ± 0.58	6 ± 0.00	14 ± 2.83	33.1
A. ustus	12.3 ± 0.58	10 ± 0.00	8 ± 1.00	6 ± 0.00	0 ± 0.00	15 ± 0.00	66.3
A. terreus	11.7 ± 0.58	9 ± 0.00	7.3 ± 0.58	6 ± 0.00	0 ± 0.00	17 ± 1.41	66.3
A. tamarii	17.7 ± 0.58	14.3 ± 0.58	7.7 ± 0.58	6 ± 0.00	0 ± 0.00	12 ± 0.00	66.3

A total of sixteen species of *Fusarium* were tested for antifungal activity out of which four namely; *F. proliferatum*, *F. sporotrichoides*, *F. graminearum* and *F. moniliforme* showed the best activity with a similar MIC of 33.1 mg/ml. The most resistant species was *F. lateritium* with an MIC of 265 mg/ml. The rest showed moderate antifungal activity with MIC values ranging from 66.3 to 132.5 mg/ml.

The highest resistance against the oil was observed among the *Penicillium* species. Out of the fourteen species tested, seven of them namely *P. citrinum*, *P. cyclopium*, *P. expansum*, *P. paxilli*, *P. ochraceum*, *P. digitatum* and *P. isladicum* showed a high resistance with an MIC of 265 mg/ml and generally low inhibition zones. The highest antifungal activity among the *Penicillium* species was observed against *P. claviforme* but with a low inhibition zone.

DISCUSSION

The antifungal activity of the *P. capense* essential oil is due to the presence of several components known to have biological activities. Although essential oils are complex mixtures of naturally occurring compounds that are predominantly monoterpenes and sesquiterpenes, it is the monoterpenoid fraction that accounts for most antimicrobial activity. Being small molecules, they diffuse into and damage cell membrane structures (Sikkema et al., 1995). α–Pinene and β–pinene, which were found in appreciable amounts in the oil under study, are known to show antifungal activity (Magiatis et al., 1999). Wilson et al. (1997) showed that myrecene, α-pinene and β-pinene were associated with antifungal activity against *Botrytis cinera*. The other monoterpenoids present in appreciable amounts and reported to show antifungal activities are limonene (Chee et al., 2009) and linalool (Pattnaik et al., 1997). Linalool has also been known to inhibit spore germination. The inhibition of sporulation appeared to arise from respiratory suppression of aerial mycelia (Lahlou and Berrada, 2001).

Table 3: Antifungal activity of *P. capense* oil on sixteen *Fusarium* species after seven days of inoculation.

Fungus	**Inhibition zones (mm)**						**MIC mg/ml**
	Essential oil, µg x 10^2					**Nystatin, µg**	
	106.00	**53.00**	**26.50**	**13.25**	**6.63**	**100**	
F. avenaceum	14.3 ± 0.58	9.7 ± 0.58	9.7 ± 0.58	6.3 ± 0.58	0 ± 0.00	14 ± 0.00	66.3
F. oxysporum	11.7 ± 0.58	8.7 ± 0.58	6.3 ± 0.58	6 ± 0.00	0 ± 0.00	11 ± 1.41	66.3
F. proliferatum	17.3 ± 0.58	11.7 ± 0.58	11 ± 0.58	9 ± 0.00	6 ± 0.00	10 ± 1.41	33.1
F. merismoides	9.3 ± 0.58	8 ± 0.00	6 ± 0.00	0 ± 0.00	0 ± 0.00	13.5 ± 0.71	132.5
F. solani	14.7 ± 0.58	10.3 ± 0.58	6.3 ± 0.58	0 ± 0.00	0 ± 0.00	28 ± 1.41	132.5
F. chlamydosporum	7.7 ± 0.58	6.7 ± 0.58	6.3 ± 0.58	0 ± 0.00	0 ± 0.00	11 ± 1.41	132.5
F. subglutinans	13.7 ± 0.58	10 ± 0.00	8 ± 0.00	6 ± 0.00	0 ± 0.00	19 ±0.00	66.3
F. semitectum	10.7 ± 0.58	9 ± 0.00	7 ± 0.00	6 ± 0.00	0 ± 0.00	21 ± 2.12	66.3
F. nivale	15.3 ± 0.58	10.3 ± 0.58	8 ± 0.00	6 ± 0.00	0 ± 0.00	9 ± 0.00	66.3
F. culmorum	10.3 ± 0.58	8.3 ± 0.58	7 ± 0.00	6 ± 0.00	0 ± 0.00	12 ± 1.41	66.3
F. lateritium	7.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	15.5 ± 0.71	265
F. scirpi	9.7 ± 0.58	7.3 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	20.5 ± 0.71	132.5
F. sporotrichoides	13.3 ± 0.58	11.3 ± 0.58	9.7 ± 0.58	7.7 ± 0.58	6 ± 0.00	12 ± 0.00	33.1
F. crookwellence	16.3 ± 0.58	9.7 ± 0.58	7.7 ± 0.58	6 ± 0.00	0 ± 0.00	34 ± 1.41	66.3
F. graminearum	10.7 ± 1.15	8.7 ± 0.58	7.3 ± 0.58	7 ± 0.00	6 ± 0.00	10 ± 0.00	33.1
F. moniliforme	15.3 ± 0.58	9.3 ± 0.58	8 ± 0.00	7 ± 0.00	6 ± 0.00	9 ±0.00	33.1

Table 4: Antifungal activity of *P. capense* oil on fourteen *Penicillium* species after seven days of inoculation.

Fungus	Inhibition zones (mm)						MIC mg/ml
	Essential oil, µg x 10^2					Nystatin, µg	
	106.00	53.00	26.50	13.25	6.63	100	
P. citrinum	19.7 ± 1.53	6 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	17 ± 1.41	265
P. rubrum	27 ± 1.00	6.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	19 ± 0.00	132.5
P. viridicatum	10.7 ± 1.15	8.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	17.5 ± 0.71	132.5
P. rugulosum	20.3 ± 0.58	6.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	16 ± 0.00	132.5
P. cyclopium	8.3 ± 0.58	6.7 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	19 ± 0.00	265
P. expansum	11.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	17.5 ± 0.71	265
P. claviforme	9.7 ± 0.58	8.3 ± 0.58	7 ± 0.00	6.7 ± 0.58	6 ± 0.00	17 ± 1.41	33.1
P. paxilli	9.3 ± 0.58	7 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	15 ± 0.00	265
P. ochraceum	11 ± 1.00	6 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	15 ± 0.00	265
P. digitatum	13.3 ± 2.08	6 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	18.5 ± 0.71	265
P. palitans	12.3 ± 0.58	9.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	15.5 ± 0.71	132.5
P. wortmanni	9.7 ± 0.58	7.3 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	18 ± 0.00	132.5
P. isladicum	7 ± 0.00	6.3 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	15 ± 0.00	265
P. purpurogenum	18.7 ± 0.58	9.7 ± 0.58	6 ± 0.00	0 ± 0.00	0 ± 0.00	17.5 ± 0.00	132.5

Sesquiterpenes have also been reported to show various antimicrobial activities (Barrero et al., 2005). The major sesquiterpene constituent in the oil δ–cadinene has been reported (Cheng et al., 2005) to be the main compound of the antifungal essential oil of Japanese cedar *Cryptomeria japonica*. Byclogermacrene which was found in low amounts in the oil is known to show relatively good antifungal activity (Siqueira et al., 2011). It is the precursor of spathulenol, an oxygenated sesquiterpene which was also present in the oil. The antimicrobial activity of caryophyllene oxide which is also a constituent of this oil has also been reported (Ulubelen et al., 1994). Tellez et al. (2000) also reported that α-humulene has antifungal activity. Sesquiterpenes such as copaene and germacrene D have also been reported to be antifungal (Gallori et al., 2001). The antifungal activity of the oil can also be associated with the presence of arylpropanoids, whose antimicrobial activity has been patented indicating their importance (Maxia et al., 2012).

The observed antifungal activities of the oil may also be due to the synergistic effects of most of its constituents. Several mechanisms by which mycelia growth may be reduced or totally inhibited have been proposed. It is generally accepted that it is the toxic effects of the essential oil components on the functionality and structure of the cell membrane that is responsible for the activity (Knobloch et al., 1988). Sharma and Tripathi (2008) reported that essential oils act on the hyphae of the mycelium, provoking the exit of components from the cytoplasm, the loss of rigidity of the hyphae cell wall and resulting in its collapse.

Conclusion

The essential oil of *P. capense* possesses fungicidal properties. It can be used in the preparation of antifungal products that can inhibit the growth of *Aspergillus*, *Fusarium* and *Penicillium* moulds on foods and in the process control the production of mycotoxins. The oil can be used after an evaluation of its toxicity.

ACKNOWLEDGEMENTS

The authors are grateful to the Lake Victoria Basin Research Initiative (VicRes) for the financial support and Prof. Uzi Ravid of Newe Yaár Centre in Israel for making available the GC – MS apparatus for this work.

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